Exhaled hydrogen peroxide correlates with the release of reactive oxygen species by blood phagocytes in healthy subjects

U. SZKUDLAREK*, Ł. MARIA*, M. KASIELSKI†, S. KAUCKA‡ AND D. NOWAK*

*Department of Experimental and Clinical Physiology, Institute of Physiology and Bichemistry, Medical University of Lodz, Mazowiecka str 6/8, 92-215 Lodz, Poland †Practical Clinical Training Centre, Medical University of Lodz, Lodz, Poland ‡Department of Family Medicine, Medical University of Lodz, Lodz, Poland

Abstract Various cells including polymorphonuclear leukocytes, alveolar macrophages and type-II pneumocytes may be a source of exhaled hydrogen peroxide (H_2O_2) in airways of humans. H_2O_2 can convert into hydroxyl radicals leading to peroxidative damage of airways structures and formation of volatile thiobarbituric acid-reactive substances (TBARs). We tested whether exhalation of H_2O_2 and TBARs by healthy subjects depends on reactive oxygen species generation from blood phagocytes. The expired breath condensate (EBC) and blood specimens were collected from 41 healthy, never smoked subjects (mean age 20.7 $\pm$0.8 years, 18 men, 23 women) and then the EBC concentration of H_2O_2 and TBARs and 2×10^{-5} M fMLP-provoked whole blood chemiluminescence response was measured. The mean concentration of H_2O_2 and TBARs in EBC was 0.28 $\pm$0.17 and 0.04 $\pm$0.13 μM with ratio of positive readings reaching 36/41 and 4/41, respectively. The chemiluminescence response to n-formyl-methionyl-leveyl-phenylalanine stimulation was obtained in all cases and the following parameters were estimated : basal chemiluminescence (bCl) ; peak chemiluminescence (pCl); absolute light emission (aCl); and peaktime. H_2O_2 levels in EBC positively correlated (Spearmann test) with bCl (r=0.41, $P<0.01$), pCl (r=0.47, $P<0.01$), aCl (r=0.49, $P<0.001$), peaktime (r=0.52, $P<0.001$) in the whole group and with bCl (r=0.56, $P<0.01$), pCl (r=0.67, $P<0.01$), aCl (r=0.66, $P<0.01$) in men and with aCl (r=0.41, $P<0.05$) and peaktime (r=0.48, $P<0.05$) in women. No association between exhaled TBARs and blood phagocytes activity was found. These results indicate that H_2O_2 exhalation in healthy never smoked subjects depends on ability of blood phagocytes to generate reactive oxygen species.

doi:10.1053/rmed.2003.1506, available online at http://www.sciencedirect.com

Keywords hydrogen peroxide; thiobarbituric acid reactive substances; expired breath condensate; whole blood chemiluminescence; phagocytes; polymorphonuclear leukocytes; reactive oxygen species.

INTRODUCTION

Human respiratory tract contains variety of cells that are able to release reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (H_2O_2). These include polymorphonuclear leukocytes (PMNs), alveolar macrophages, mastocytes and type-II pneumocytes (1–3). H_2O_2 in the presence of iron or copper ions can convert into hydroxyl radicals (4) and induce peroxidative damage to various biomolecules leading to generation of volatile thiobarbituric acid reactive substances (TBARs) (5,6). Part of H_2O_2 not decomposed by antioxidant enzymes and not entering free radical-mediated processes can evaporate from the surface of epithelial lining fluid being exhaled with expiratory air. Healthy subjects exhale detectable levels of H_2O_2 and occasionally TBARs that are rised by cigarette smoking and chronic inflammatory processes in the airways (7–12). Thus exhaled H_2O_2 and also TBARs may serve as one of non-invasive markers of airways inflammation and oxidative stress. There is indirect evidence that H_2O_2 exhalation is associated with the number and activity of phagocytes in airways of patients with lung inflammatory disorders. In asthmatics the concentration of H_2O_2 in expired breathe condensate (EBC) positively correlated with intracellular calcium rise in stimulated PMNs (13) and the number of eosinophils in induced sputum (14). Also, H_2O_2 levels in EBC of patients with adult respiratory distress syndrome correlated with plasma lysozyme that reflects in vivo neutrophil turnover (15). In

Received 19 February 2002, accepted in revised form 11 October 2002
Correspondence should be addressed to. Dariusz Nowak, Department of Experimental and Clinical Physiology, Institute of Physiology and Biochemistry, Medical University of Lodz, Mazowiecka str 6/8, 92-215 Lodz, Poland. Phone/Fax +48(42)678-2661
E-mail : nowak@toya.net.pl.

addition, inhaled steroids that suppress inflammatory cells activity decreased H_2O_2 exhalation in children and adult patients with asthma (16,17). However, no studies devoted to direct association between phagocytes ability to produce ROS and exhalation of H_2O_2 and TBARs by healthy subjects have been published so far.

The number and activity of phagocytes in respiratory tract of patients with chronic airways inflammation (e.g. bronchial asthma, chronic obstructive pulmonary disease) is higher than that found in healthy subjects (18,19). This suggests that contribution of other H_2O_2 sources like alveolar epithelial cells to total pool of exhaled H_2O_2 may be higher in healthy subjects than in these patients. On the other hand, type-II pneumocytes and phagocytes use probably the same NADPH oxidase-dependent pathways for ROS generation (2,3,20). Therefore, the purpose of this study was to investigate whether H_2O_2 and TBARs exhalation by healthy subjects depends on ability of blood phagocytes to generate ROS after stimulation with chemotactic peptide *n*-formyl-methionyl-leucyl-phenylalanine (fMLP) in a whole blood chemiluminescence assay. We found that concentration of H_2O_2 in EBC collected from healthy never smoked subjects positively correlated with the intensity of blood phagocytes chemiluminescence response to fMLP.

MATERIAL AND METHODS

Reagents

Dimethyl sulfoxide (DMSO) and thiobarbituric acid (TBA), peroxidase from horseradish type-II (HRP), homovanillic acid, fMLP, luminol and butylated hydroxytoluene were purchased from Sigma (St. Louis, MO, USA). Sterile Ringer solution (pH 7.4) was from Biomed (Lublin, Poland). All other reagents were obtained from POCH (Gliwice, Poland) and were of analytical grade. fMLP was dissolved in DMSO to a final concentration 2×10^{-2} M and stored at -80°C until assay. This solution was diluted with sterile 0.9% NaCl to fMLP concentration 4×10^{-4} M just before the use. Aqueous solution of HRP (10 U/ml^{-1}) and homovanillic acid (4 mM) were prepared freshly before the assay. Luminol solution was prepared by dissolving 25 mg luminol in 90 ml 0.1 M Na_2HPO_4 and then pH was adjusted to 7.4 with 1 N HCl and volume filled up to 100 ml with distilled water (21). Afterwards the solution was filtered through 0.2 μM Millipore filter and stored at 4°C in the dark for not longer than 2 weeks. TBA solution was prepared by dissolving 0.67 g TBA in 100 ml deionized water and then diluted 1:1 with glacial acetic acid. Sterile, deionized water (resistance > 18 MΩcm, HPLC Water Purification System USF ELGA, England) was used throughout the study.

Study population

Forty-one never smoked healthy volunteers (18 men and 23 women), students of the second year of Medical Faculty of Medical University were included in the study (Table I). They were free of any medication, had not suffered from any infectious diseases for 3 months prior the study and routine physical examination showed nothing abnormal. No women was pregnant nor used oral contraceptives.

Study design

Subjects were asked to attend the laboratory between 8°° and 10°° for EBC collection. Then the spirometry was performed with Lungtest 1000 spirometer (MES S.c., Kraków, Poland) equipped with software compatible to American Thoracic Society standards (22) and 10 ml aliquots of blood were drawn into EDTA K3 Vacuette and Z Serum Clot Activator Vacuette tubes (Greiner Labor technik) for evaluation of resting and fMLP-induced whole blood chemiluminescence and determination of serum TBARs levels. All healthy volunteers involved in the study were given informed consent and the study protocol was approved by Ethics Committee of Medical University of Lodz.

Collection of expired breath condensate

The collecting device consisted of a mouthpiece with saliva trap connected to a glass Liebig tubecooler (cooling

Table I. Characteristic of study subjects.

	Whole group	Men	Women
n	41	18	23
Age	20.7 ± 0.8	20.6 ± 0.8	20.8 ± 0.8
BMI	21.7 ± 2.3	23.3 ± 2.3	20.5 ± 1.6
FEV_1 %	96.6 ± 4.2	101.7 ± 6.7	90.9 ± 3.8
FEV_1/FVC %	94.8 ± 3.9	91.3 ± 4.2	95.8 ± 4.1
FVC %	104.8 ± 4.3	109.8 ± 4.1	100.9 ± 4.6
Erythrocytes × $10^6/\mu$l	4.4 ± 0.7	4.7 ± 0.9	4.1 ± 0.7
Hemoglobin g/dl	13.9 ± 1	14.9 ± 3.4	13.0 ± 2.5
WBC × $10^3/\mu$l	6.2 ± 2.1	6.0 ± 1.7	6.2 ± 2.1
PMNs × $10^3/\mu$l	4.3 ± 1.5	4.1 ± 1.4	4.7 ± 1.6
Monocytes × $10^3/\mu$l	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.2

BMI—body mass index, FVC—forced vital capacity, FEV_1—forced expiratory volume in the first second, WBC—white blood cells, PMNs—polymorphonuclear leukocytes, TBARs—thiobarbituric acid reactive substances. FEV_1, FVC, FEV_1/FVC are expressed as percent of predicted value. The predicted values were derived from the European Community for Steel and Coal statement (40).

and collecting tube 55 cm length, internal diameter 10 mm, the external jacket diameter 36 mm) (Labmed, Lodz, Poland cat no 6010). The tube was cooled with ethanol pumped in the closed circuit and its temperature was kept at -9°C with Multi Temp III (Pharmacia Biotech). This temperature was the lowest one that allowed to collect liquid EBC in the sterile plastic tube covered with ice (Sarstedt, Numbrecht, volume 13 ml, internal diameter 14 mm) mounted at the base of Liebig tubecooler. Preliminary experiments showed that further decrease in ethanol temperature caused congelation of EBC inside the cooling tube and stopped its collection. The EBC was always collected between $8^{\circ\circ}$ and $10^{\circ\circ}$. Volunteers were asked to breathe out through a mouthpiece and to breathe in with the mouthpiece removed, for 20 min. Each subject wore a nose clip and rinsed their mouth with distilled water just before and at 7 and 14 min of collection (7,8). At the end of collection, 5–6 ml aliquots of EBC were transferred to Eppendorf tubes and stored at -80°C for not more than 7 days until H_2O_2 and TBARs measurement (8). The respiratory rate during EBC collection ranged from 17 to 23 breaths/min. Previous studies showed that 5-fold rise in respiratory frequency and minute ventilation did not change significantly the concentration of H_2O_2 in EBC (23). In addition, hyperventilation related to moderate exercise did not change significantly the H_2O_2 and TBARs exhalation in healthy subjects (7). Six subjects (four men, two women) were asked to attend two EBC collection sessions in 30 min intervals. The reproducibility for determined EBC H_2O_2 concentrations was 11.6%.

Measurement of H_2O_2 in expired breath condensate

The concentration of H_2O_2 in EBC was measured according to the method of Ruch *et al*. (24) with some modifications (8) Briefly, 600 μl of EBC was mixed with 600 μl of HRP solution (1 U/ml) containing 100 μM homovanillic acid and was incubated for 60 min at 37°C. Then, the sample was mixed with 150 μl 0.1 M glycine – NaOH buffer (pH 12.0) with addition of 25 mM EDTA and emission at 420 nm after specimen excitation at 312 nm was measured using a Perkin Elmer Luminescence Spectrometer LS-50B (Norwalk, CT, USA). Readings were converted into μM using the regression equation Y=0.012 $(X-X_0)-0.007$ (where Y=micromoles of H_2O_2 per litre of expired breath condensate; X=intensity of emission at 420 nm expressed in arbitrary units; X_0=intensity of emission given by reference sample receiving deionized water instead of EBC) (8). The lower limit of H_2O_2 detection was 0.083 μM (6). The intra-assay variability did not exceeded 2% for standard H_2O_2 solutions ranging from 0.1 to 0.5 μM.

Measurement of TBARs in expired breath condensate

The content of TBARs in EBC was determined as previously described (8). Briefly, 100 μl of the condensate was mixed with 2 ml of TBA solution, boiled for 30 min, allowed to cool in ambient temperature and then chromogen was extracted into 2.5 ml of butanol by vigorous shaking for 1 min. Following centrifugation (10 min, $1500 \times g$, 25°C), TBARs were measured spectrofluorimetrically, excitation at 515 nm, emission at 546 nm (25). Readings were converted into μM using the regression equation Y=0.39 $(X-X_0)$-1.32 (where Y=micromoles of TBARs per liter of EBC; X=intensity of emission at 546 nm expressed in arbitrary units; X_0=intensity of emission given by reference sample receiving deionized water instead of EBC). Tetramethoxypropane (0.01–50 μM) was used as an external standard and the method sensitivity was 0.05 μM (8). The intra-assay variability did not exceeded 3% for 0.1 and 0.5 μM tetramethoxypropane solution.

Measurement of TBARs in serum

Serum TBARs levels were determined as previously described (26). Briefly, 0.5 ml of 0.05 M H_2SO_4 and 0.25 ml of 1.23 M trichloroacetic acid were added to 0.05 ml of serum, mixed and then centrifuged for 10 min (1500g, 4°C). The supernatant was discarded and mixed with 2 ml of distilled water, 0.01 ml of 4.53 nM butylated hydroxytoluene in methanol and 0.5 ml of TBA solution. The obtained mixture was incubated for 30 min in a water bath at 100°C in tightly closed tubes. After cooling, 2.5 ml of butanol was added to the tubes, and after intensive shaking centrifuged for 10 min (1500g, 20°C). Measurements were made in the supernatant of butanol layer. Fluorescence was measured at an excitation wavelength of 515 nm and an emission wavelength of 546 nm. Readings were converted into μM using the calibration curve obtained for tetramethoxypropane (0.01–50 μM). The control contained deionized water instead of serum.

Whole blood chemiluminescence assay

The resting and fMLP-induced whole blood chemiluminescence (Cl) was measured according to the method of Kukovetz *et al*. (21) Venous blood was drawn into sterile vacuette with EDTA just after EBC collection. Then 3 μl blood specimen was added to 947 μl of mixture solution placed in the 1251 luminometer (Bio-Orbit®, Turku,Finland) and incubated for 30 min at 37°C. Afterwards the resting luminescence was recorded continuously for 1 min and then 50 μl fMLP solution was added by an automatic dispenser to final concentration of 2×10^{-5} M and measurement was continued for the further 7 min. The following parameters were calculated:

basal chemiluminescence (bCl), peak chemiluminescence (pCl—maximal Cl signal reached after fMLP addition), absolute light emission from 3 μl of tested whole blood specimen after addition of fMLP (aCl—area under the CL intensity curve after fMLP addition till returning to baseline) and peaktime (time from fMLP addition to appearance of maximal Cl signal) (Fig. 1). In all cases the Cl signal returned to baseline between 6th and 8th min of recording. Individual results were obtained from triplicate experiments and bCl and pCl were expressed in arbitrary units (aU), while aCl was expressed in aU × seconds (aU × s). The intra-assay variation was 3.7% , 2.3 % , 9.8% and 4.7% (n=6) for bCl , aCl, peaktime and pCl , respectively. The mixture solution was prepared freshly before the assay by adding 1 ml Ringer solution, 5 ml 1.41 μM luminol solution, 0.2 ml 277.5 mM glucose solution to 3.6 ml distilled water (2l). Blood cell count was performed with ABX MICROS analyser (AVL, Montpellier France).

Statistical analysis

Data are expressed as mean ± SD. For readings that gave results below the method sensitivity the H_2O_2 and TBARs concentration in EBC was assumed 0 nM. The differences between groups were computed with Mann–Whitney *U*-test or Friedman ANOVA test according to the sample distribution. The differences between appropriate ratios of positive H_2O_2 and TBARs readings were determined with chi-square test. Correlation coefficients were calculated by the Spearmann test. In all cases a P value of < 0.05 was considered significant.

RESULTS

The concentration of H_2O_2 and TBARs in EBC of healthy never smoked subjects was 0.28 ± 0.17 μM and 0.04 ± 0.13 μM. There were no differences in exhalation of these compounds between men and women (Table 2). The ratio of positive H_2O_2 readings (above the method sensitivity) in the whole group was much higher than that for TBARs (36/41 *vs*. 4/41 , P=0.0001). In opposite to EBC specimens the majority of serum samples was positive for TBARs (4/41 *vs*. 28/41, P=0.001) and their mean concentrations reached 0.93 ± 1.25 μM for the whole group, 1.04 ± 1.60 μM for men and 0.84 ± 0.93 μM for women. The generation of ROS by circulating phagocytes (PMNs and monocytes) was evaluated by means of the whole blood chemiluminescence assay. Phagocytes were stimulated by fMLP addition and in all cases the Cl response was obtained. Four parameters of Cl response were measured and no significant effect of sex on ROS generation in the whole blood assay was noted (Table 3). Both, pCl and aCl correlated with WBC count (r=0.38, 0.34, $P < 0.05$) and blood phagocytes (PMNs and monocytes) count (r=0.33, 031, $P < 0.05$). Similar correlation coefficients may result from strong correlation between WBC count and blood phagocytes count (r=0.95, $P < 0.0001$). Analysis of correlations of EBC

TABLE 2. H_2O_2 and TBARs concentrations in expired breath condensate of healthy subjects.

Subjects	H_2O_2 (μM)	TBARs (μM)
Whole roup	0.28 ± 0.17	0.04 ± 0.13
Men	0.33 ± 0.19	0.07 ± 0.18
Women	0.24 ± 0.14	0.01 ± 0.04

Specimens of expired breath condensate were collected between 8°° and 10°° and H_2O_2 and TBARs concentrations were determined fluorimetrically with homovanillic and thiobarbituric acid method, respectively.

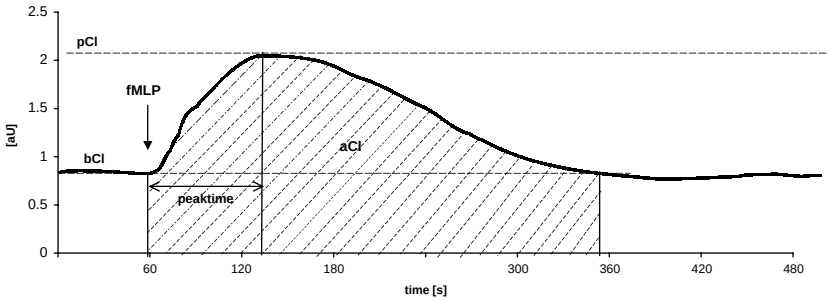

FIG. 1. Typical curve of whole blood chemiluminescence assay. Three micro liters of blood were mixed with 947 μl of mixture buffer and placed in luminometer. Baseline Cl was recorded over 1 min and then fMLP to final concentration 2×10^{-5} M was added (arrow). bCl—basal chemiluminescence; pCl—peak chemiluminescence (maximal Cl signal reached after fMLP addition); aCl—total light emission after addition of fMLP (area under the CL intensity curve after fMLP addition till returning to baseline); peaktime—time from fMLP addition to pCl appearance. All parameters except of peaktime and aCl were expressed in arbitrary units. Peaktime and aCl were expressed in seconds and arbitrary units × seconds.

Table 3. Parameters of fMLP-induced whole blood chemiluminescence response in healthy subjects.

Cl parameter	Whole group	Men	Women
bCl aU	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.3
pCl aU	1.7 ± 1.1	1.9 ± 1.6	1.5 ± 0.6
aCl aU × s	559.1 ± 271.3	607.4 ± 365.5	516.5 ± 153.2
Peaktime s	198 ± 56	224 ± 45	172 ± 66

Cl—chemiluminescence; bCl—basal chemiluminescence; pCl—peak chemiluminescence after fMLP addition; aCl—absolute light emission from 3 μl of assayed whole blood specimen after addition of fMLP; peaktime—time from fMLP addition to pCl appearance; aU—arbitrary units; No significant differences between men and women were found.

Table 4. Correlations (r) between exhaled H_2O_2 and parameters of whole blood chemiluminescence response in healthy subjects.

Cl parameters	H_2O_2 level in expired breath condensate		
	Whole group	Men	Women
bCl	0.41**	0.56**	0.36
pCl	0.47**	0.67**	0.35
aCl	0.49***	0.66*	0.41*
peaktime	0.52***	0.37	0.48*

Cl—chemiluminescence; bCl—basal chemiluminescence; pCl—peak chemiluminescence after fMLP addition; aCl—absolute light emission from 3 μl of whole blood after addition of fMLP; peaktime—time from fMLP addition to pCl appearance.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

H_2O_2 levels with parameters of fMLP-induced Cl response showed many statistically significant results (Table 4). Exhaled H_2O_2 positively correlated with bCl (Fig. 2), pCl, aCl (Fig 3) and peaktime in the whole group of healthy volunteers. There was no significant correlation between EBC H_2O_2 concentrations and WBC (r=0.05, P=0.75) and blood phagocytes count (r=0.06, P=0.70). In man subgroup the levels of H_2O_2 correlated with bCl, pCl, aCl while in the women the significant association between exhaled H_2O_2 and aCl, and peaktime was found (Table 4). No significant correlations were observed between exhaled and serum TBARs and WBC count , blood phagocytes count and parameters of fMLP-induced Cl response (data not shown).

DISCUSSION

We found that exhalation of H_2O_2 by healthy never smoked subjects correlates with the ability of blood phagocytes to produce ROS as determined by the whole blood chemiluminescence assay. The positive correlation of EBC H_2O_2 levels with resting production of ROS (bCl) as well as with parameters describing the fMLP-induced ROS generation (pCl, aCl , peaktime) was found. The mean number of monocytes was 14-fold lower than number of PMNs in assayed blood specimens (Table 1). Therefore, PMNs seem the main source of ROS including H_2O_2 in the whole blood chemiluminescence assay. It suggests that exhaled H_2O_2 mainly derives from PMNs that migrate from circulation into airways of healthy subjects. This conclusion is surprising in the light of airways anatomical conditions where PMNs compose the minority of phagocytes capable to produce and release H_2O_2. Normally, alveolar macrophages contribute to 80–90% whilst PMNs to 1–4% of bronchoalveloar lavage fluid total cell count, respectively (27). Alveolar type-II cells are also able to generate H_2O_2 (2,3). Although, in vitro type-II cells produce 4.4 times less H_2O_2 than phagocytes (3) their high number representing about 15% of the total cell lung population (28) make them important potential source of H_2O_2 in the airways. On the other hand, alveolar macrophages release catalase that is recognized as the main antioxidant macromolecule of epithelial lining fluid (29). Similarly, alveolar type-II cells decompose H_2O_2 by catalase-dependent mechanisms (30,31) and their intracellular catalase load is 4.5 -fold higher than that of alveolar macrophages (3). Thus catalase activity derived from both alveolar macrophages and type-II cells may diminish their net contribution as sources of exhaled H_2O_2 in healthy subjects. This is consistent with the observation that PMNs but not alveolar macrophages are responsible for the rise in ROS production in airways of healthy volunteers after exposition to ozone (32). These may explain the positive correlation of H_2O_2 levels in EBC with activity of blood PMNs although these cells are not so numerous as alveolar macrophages or type-II pneumocytes in airways of healthy subjects. It should be noted that PMNs primary granules also contain catalase, (33) however, its contribution to antioxidant screen of epithelial lining fluid seems low (29).

Human plasma contains detectable amounts of H_2O_2 (4). It cannot be excluded that H_2O_2 released from circulatory phagocytes may diffuse from blood streem through pulmonary endothelium into lower airways and evaporate from epithelial lining fluid.

This hypothesis may clearly explain the association between EBC H_2O_2 levels and activity of blood phagocytes. However, plasma contains variety of compounds that decompose H_2O_2 (4). In addition, erythrocytes are also able to rapidly scavenge H_2O_2 (34) and endothelium of pulmonary vessels and airways epithelium contains antioxidants that may suppress the H_2O_2 flow from blood into lungs in healthy subjects. The dependency of EBC

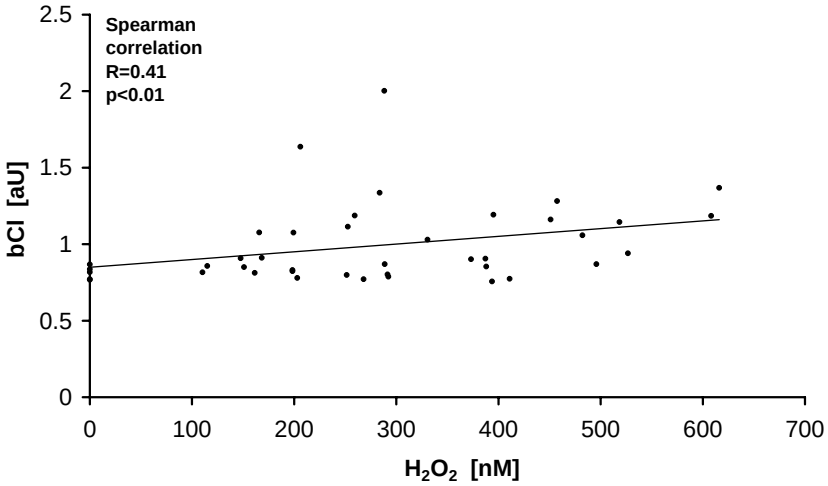

Fig. 2. Positive correlation between H_2O_2 levels in expired breath condensate of healthy never smoked subjects and basal chemiluminescence (bCl) of whole blood.

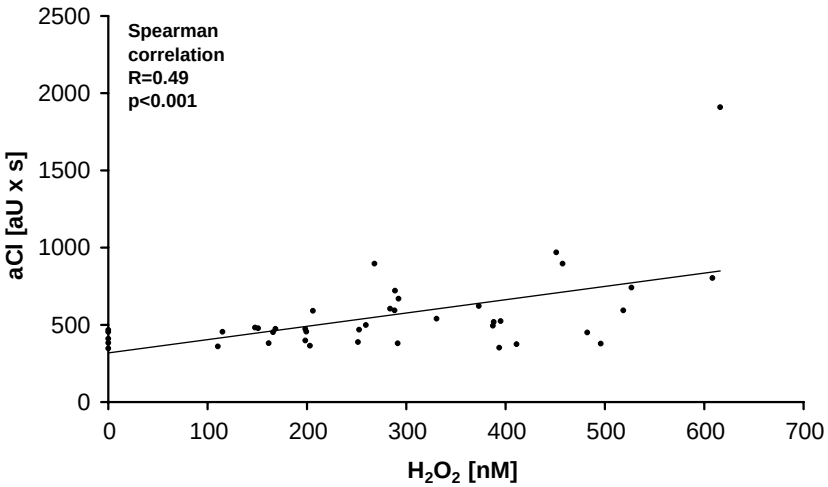

Fig. 3. Positive correlation between H_2O_2 concentrations in expired breath condensate of healthy never smoked subjects and absolute light emission (aCl) from 3 μl of assayed whole blood specimen after addition of fMLP.

H_2O_2 levels on expiratory flow rate (35) suggests important airways contribution to exhaled H_2O_2 . Since airways epithelial cells did not produce large amounts of ROS the diffusion of H_2O_2 from blood into airways could be one of possible explanation. On the otherhand, the EBC collection at higher expiratory flow rate resulted in the decrease in H_2O_2 levels (35). Under these conditions more droplets of airways respiratory fluid are collected in cooled condenser (36). This argues against hypothesis that airways are more important source of exhaled H_2O_2 than alveoli.

Alveolar macrophages and PMNs generate superoxide radical and H_2O_2 via the protein kinase C-activated NADPH oxidase (37). Recent studies showed that type-II pneumocytes use the plasma membrane bound NADPH oxidase-like system for ROS production. Its activation was induced by variety of agonists (arachidonic acid, zymosan, bacteria) known as factors evoking H_2O_2 response in phagocytes and was blocked by protein kinase C inhibitors (20). These suggest that majority of H_2O_2 evaporating from lower airways surface is a product of NADPH oxidase activity independently of its cell origin. It is possible that regulation of NADPH oxidase activity is similar in phagocytes and type-II cells (20,37). Thus blood PMNs ability to produce ROS may reflect H_2O_2 release from cells localized in the close neighborhood of lower airways epithelial lining fluid. This may be another explanation of positive association between exhaled H_2O_2 and ROS production in the whole blood system. Other sources like mitochondria of lung cells,

arachidonic acid metabolism, xanthine oxidase activity may also contribute to exhaled H_2O_2 (4,1). However, they probably have importance in subjects with lung inflammatory processes or those exposed to hyperoxia (1).

No association was found between ROS production by blood phagocytes and exhaled and serum TBARs. These may result from high ratio of negative TBARs readings in EBC and from sufficient serum antioxidant defence to neutralize ROS derived from phagocytes. Perhaps, in healthy subjects without any inflammatory processes the baseline ROS production by circulating phagocytes is too low to induce peroxidative damage to plasma biolmolecules with subsequent TBARs generation**.** Negative EBC TBARs readings in subjects with detectable serum TBARs indicate that these end products of lipid peroxidation did not diffuse from blood into airways epithelial lining fluid.

We did not observe any significant effect of sex on resting and fMLP-provoked light emission in the whole blood chemiluminescence assay. This is consistent with results of previous studies showing no differences between men and women in respect of ROS production in the whole blood system (21,38). We also did not find significant differences in H_2O_2 EBC levels between men and women. This differs from results of our previous study showing higher H_2O_2 exhalation in women (7). Our present study involved almost 2 times younger subjects and this may be a causation of different results.

In conclusion we found that H_2O_2 exhalation by healthy subjects depends on ability of blood phagocytes to produce ROS. This clearly shows phagocytes as important source of exhaled H_2O_2. This finding may also be helpful for understanding the phenomenon of increased H_2O_2 exhalation in patients without pulmonary pathology but with systemic processes that enhance activity of circulating phagocytes, e.g. subjects with severe brain injury without pulmonary infiltrates (23) or those with polytrauma not involving the chest (39).

Acknowledgements

The authors wish to thank Mrs. Zdzisawa Sêdziñska and Mrs. Anna Kliszko for technical assistance during the experiments. The study was supported by a grant 502-11-649 (102) from Medical University, Lodz, Poland.

REFERENCES

1. Repine JE, Bast A, Lankhorst I. Oxidative stress in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1997; **156:** 341–357.
2. Piotrowski WJ, Marczak J, Dinsdale D, Kurmanowska Z, Tarasow Y, Komos J, Nowak D. Release of hydrogen peroxide by rat type II pneumocytes in the prolonged culture. *Toxicol In Vitro* 2000; **14:** 85–93.
3. Kinnula VL, Everitt JI, Whorton AR, Crapo JD. Hydrogen peroxide production by alveolar type II cells, alveolar macrophages, and endothelial cells. *Am J Physiol* 1991; **261:** L84–91.
4. Halliwell B, Clement MV, Long L.H. Hydrogen peroxide in the human body. *FEBS Letters* 2000; **486:** 10–13.
5. Pryor WA. Oxy-radicals and related species: their formation lifetimes and reactions. *Ann Rev Physiol* 1986; **48:** 657–667.
6. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biol Med* 1990; **9:** 515–540.
7. Nowak D, Kaucka S, Biaasiewicz P, Król M. Exhalation of H_2O_2 and thiobarbituric acid reactive substances (TBARs) by healthy subjects. *Free Radical Biol. Med.* 2001; **30:** 178–186.
8. Nowak D, Kasielski M, Antczak A, Pietras T, Bialasiewicz P. Increased content of thiobarbituric acid reactive substances and hydrogen peroxide in the expired breath condensate of patients with stable chronic obstructive pulmonary disease. No significant effect of cigarette smoking. *Respir Med* 1999; **93:** 389–396.
9. Nowak D, Antczak A, Król M, Pietras T, Shariati B, Bialasiewicz P, Jeczkowski K, Kula P. Increased content of hydrogen peroxide in expired breath of cigarette smokers. *Eur Respir J* 1996; **9:** 652–657.
10. Antczak A, Nowak D, Shariati B, Król M, Kurmanowska Z. Increased hydrogen peroxide and TBA-reactive products in expired breath condensate of asthmatic patients. *Eur Respir J* 1997; **10:** 1235–1241.
11. Dohlman A, Black HR, Royall JA. Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patients with asthma. *Am Rev Respir Dis* 1993; **148:** 955–960.
12. Dekhuijzen PNR, Aben KKH, Dekker I, Aarts LPHJ, Wielders PLML, Van Herwaarden CLA, Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996; **154:** 813–816.
13. Antczak A, Nowak D, Biaasiewicz P, Kasielski M. Hydrogen peroxide in expired air condensate correlates positively with early steps of peripheral neutrophil activation in asthmatic patients. *Arch Immunol Ther Exp* 1999; **47:** 119–126.
14. Horvath I, Donnelly LE, Kiss A, Kharitonov SA, Lim S, Chung KF, Barnes PJ. Combined use of exhaled hydrogen peroxide and nitric oxide in monitoring asthma. *Am J Respir Crit Care Med* 1998; **158:** 1042–1046.
15. Baldwin SR, Simon RH, Grum CM, Ketai LH, Boxer LH, Devall LJ. Oxidant activity in expired breath of patients with adult respiratory distress syndrome. *Lancet* 1986; **I:** 11–14.
16. Jobsis Q, Raatgeep HC, Hermans PWM, de Jongste JC. Hydrogen peroxide in exhaled air is increased in stable asthmatic children. *Eur Respir J* 1997; **10:** 519–521.
17. Antczak A, Kurmanowska Z, Kasielski M, Nowak D. Inhaled glucocorticosteroids decrease hydrogen peroxide level in expired air condensate in asthmatic patients. *Respir Med* 2000; **94:** 416–421.
18. Thompson AB, Daughton D, Robbins RA, Ghafouri MA, Oehlerking M, Rennard SI. Intramural airway inflammation in chronic bronchitis: characterization and correlation with clinical parameters. *Am Rev Respir Dis* 1989; **140:** 1527–1537.
19. Keatings VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-A in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996; **153:** 530–534.
20. Klaveren RJ, Roelant C, Boogaerts M, Demedts M, Nemery B. Involvement of an NAD(P)H oxidase-like enzyme in superoxide anion and hydrogen peroxide generation by rat type II cells. *Thorax* 1997; **52:** 465–471.
21. Kukovetz EM, Bratschitsch G, Hofer HP, Egger G, Schaur RJ. Influence of age on the release of reactive oxygen species by

phagocytes as measured by a whole blood chemiluminescence assay. *Free Radical Biol Med* 1997; **22:** 433–438.
22. American Thoracic Society. Standardization of spirometry 1987 update. *Am Rev Respir Dis* 1987; **136:** 1285–1296.
23. Sznajder JI, Fraiman A, Hall JB, Sanders W, Schmidt G, Crawford G, Nahum A, Factor P, Wood LDH. Increased hydrogen peroxide in the expired breath of patients with acute hypoxemic respiratory failure. *Chest* 1989; **96:** 606–612.
24. Ruch W, Cooper PH, Baggiolinii M. Assay of H_2O_2 production by macrophages and neutrophils with homovanillic acid and horseradish peroxidase. *J Immunol Meth* 1983; **63:** 347–357.
25. Yagi K. A simple fluorometric assay for lipid peroxides in blood plasma. *Biochem Med* 1986; **15:** 212–216.
26. Kasielski M. Nowak, D. Long-term administration of N-acetylcysteine decreases hydrogen peroxide exhalation in subjects with chronic obstructive pulmonary disease. *Respir Med* 2001; **95:** 448–456.
27. Drent M, Jacobs JA, Wagenaar SS. Bronchoalveolar lavage *Eur Respir Mon* 2000; **14:** 63–78.
28. Crapo JD, Young SL, Fram EK, Pinkerton KE, Barry BE, Crapo R. Morphometric characteristics of cells in the alveolar region of mammalian lungs. *Am Rev Respir Dis* 1983; **128:** S42–48.
29. Cantin AM, Fells GA, Hubbard RC, Crystal RG. Antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract. *J Clin Invest* 1990; **86:** 962–997.
30. Engstrom PC, Easterling L, Baber RR, Matalon S. Mechanisms of extracellular hydrogen peroxide clearance by alveolar type II pneumocytes. *J Appl Physiol* 1990; **69:** 2078–2084.
31. Kinnula VL, Chang L, Everitt JI, Crapo JD. Oxidants and antioxidants in alveolar epithelial type II cells: in situ, freshly isolated, and cultured cells. *Am J Physiol* 1992; **262:** L69–77.
32. Torres A, Voter KZ, Utell MJ, Whitin JC, Morrow PE, Frampton MW. Production of reactive oxygen intermediates following exposure to ozone. Relative contribution of alveolar macrophages. *Chest* 1996; **109(**suppl):8S.
33. Ballinger CA, Mendis-Handagama C, Kalmar JR, Arnold RR, Kinkade JM. Changes in the localization of catalase during differentiation of neutrophilic granulocytes. Blood 1994; **83:** 654–668.
34. Nowak D, Piasecka G. Erythrocytes protect α-1-proteinase inhibitor from oxidative inactivation induced by chemicals, myeloperoxidase - H_2O_2-halide system and stimulated polymorphonuclear leukocytes. *Exp Pathol* 1991; **42:** 47–58.
35. Schleiss MB, Holz O, Behnke M, Richter K, Magnussen H, Jorres RA. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate. *Eur Respir J* 2000; **16:** 1115–1118.
36. Effros RM, Hoagland KW, Bosbous M, Castillo D, Foss B, Dunning M, Gare M, Lin W, Sun F. Dilution of respiratory solutes in exhaled condensates. *Am J Respir Crit Care Med* 2002; **165:** 663–669.
37. Rossi F. The O_2^--forming NADPH oxidase of the phagocytes: nature, mechanism of activation and function. *Biochim Biophys Acta* 1986; **853:** 65–89.
38. Kopprasch S, Graessler J, Kohl M, Bergmann S, Schroder HE. Comparison of circulating phagocyte oxidative activity measured by chemiluminescence in whole blood and isolated polymorphonuclear leukocytes. *Clin Chim Acta* 1996; **253:** 145–57.
39. Kietzmann D, Kahl R, Muller M, Burchardi H Kettler D. Hydrogen peroxide in expired breath condensate of patients with acute respiratory failure and with ARDS. *Intensive Care Med* 1993; **19:** 78–81.
40. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report working party standardization of lung function tests. European Community for Steel and Coal. Official statement of the European Respiratory Society. *Eur Respir J* 1993; **6**(suppl. 16): 5–40.